Dependence of Superoxide Anion Production on Extracellular and Intracellular Calcium Ions and Protein Kinase C in PMA-Stimulated Bovine Neutrophils

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ABSTRACT

The involvement of both intracellular and extracellular calcium, as well as the activation of protein kinase C (PKC), in phorbol myristate acetate (PMA)-stimulated respiratory burst in bovine neutrophils has been studied. PMA significantly stimulated the superoxide anion production by these cells. The increased production of superoxide anion was inhibited by BAPTA/AM, an intracellular calcium ($[Ca^{2+}]_i$) chelator, but not affected by EGTA, an extracellular calcium ($[Ca^{2+}]_o$) chelator. PMA also induced PKC activation, and a PKC inhibitor, calphostin C, blocked the stimulatory effect of PMA on superoxide anion production by the neutrophils. Therefore, we conclude that PMA-induced respiratory burst in bovine neutrophils is $[Ca^{2+}]_i$- but not $[Ca^{2+}]_o$-dependent, and also requires PKC activation.

RÉSUMÉ

Une étude a été conduite afin d'évaluer l'implication du calcium intracellulaire et extracellulaire, ainsi que de l'activation de la protéine kinase C (PKC) sur la production de superoxyde chez les neutrophiles bovins stimulés à l'aide d'acétate de phorbol myristate acetate (APM). L'APM a provoqué la génération de superoxyde par ces cellules de façon significative. Cette production de superoxyde a été inhibée par du BAPTA/AM, un chélateur de calcium intracellulaire ($[Ca^{2+}]_i$), mais n'a pas été affectée par du EGTA, un chélateur de calcium extracellulaire ($[Ca^{2+}]_o$). De plus, l'APM a mené à l'activation de la PKC. Un inhibiteur de cette protéine, le calphostin C, a bloqué l'effet stimulant de l'APM sur la production de superoxyde des neutrophiles. Par conséquent, nous concluons que, chez les neutrophiles bovins, la génération de superoxyde induite par l'APM est dépendante du $[Ca^{2+}]_i$ mais indépendante du $[Ca^{2+}]_o$, et que l'activation de la PKC est aussi requise.

(Traduit par le docteur Serge Messier)

INTRODUCTION

The respiratory burst in neutrophils is one of the most important mechanisms for killing invading microbial pathogens. The biochemical basis for the respiratory burst is the enzyme nicotinamide adenine dinucleotide phosphate (reduced) (NADPH)-oxidase, dormant in resting cells and capable of being activated by a number of stimuli. Intracellular calcium ions (Ca^{2+}) and protein kinase C (PKC) are 2 factors believed to act as intracellular signals that trigger activation of the enzyme NADPH-oxidase in neutrophils, leading to superoxide anion (O_2^-) generation. Involvement of Ca^{2+} and PKC in the respiratory burst of human neutrophils has been extensively studied. Phorbol myristate acetate (PMA) exhibits no Ca^{2+} dependence in its stimulation of the NADPH-oxidase in human neutrophils (1,2). Also, activation of PKC has been shown to be essential for PMA-induced O_2^- generation by neutrophils from humans (3–5) and guinea pigs (6). Nevertheless, results from human neutrophils may not be extrapolated to other mammalian species such as cows since comparative studies have demonstrated substantial qualitative and quantitative variations among species (7).

PMA has been widely used in vitro as a stimulant for bovine neutrophils. However, intracellular messengers through which PMA activates bovine NADPH-oxidase remain unclear. Therefore, the purpose of the study herein was to evaluate the importance of both intra- and extracellular calcium as well as PKC in PMA-induced O_2^- production in bovine neutrophils.

MATERIALS AND METHODS

ISOLATION OF NEUTROPHILS

Neutrophils were prepared from venous blood of healthy cows as described (8). The viability and purity of neutrophils obtained were > 99% and > 95%, respectively.

MEASUREMENT OF SUPEROXIDE PRODUCTION

Generation of superoxide by neutrophils was assessed by the stimulus-induced reduction of ferricytochrome C (Sigma Chemical Co., St. Louis, MO, USA) according to the method described by Dyer et al (9) with minor modifications. After counting, neutrophils were resuspended at 2.5×10^6 cells/mL in Hank's balanced salt solution (HBSS; Gibco, Grand Island, NY, USA) containing 160 mM ferricytochrome C and activated by different doses of PMA (0.05 and 0.25 μM; Sigma). After incubation for 30 min at

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37°C, cells were removed by centrifugation at 10 000 × *g* for 45 sec. The absorbance of the supernatants was measured spectrophotometrically at 550 nm (Spectronic 600, Milton Roy, Rochester, NY, USA). The content in the reference cuvette was identical to that in the sample cuvette except for the additional presence of superoxide dismutase (SOD; 300 U/mL) (Sigma). The amounts of O_2^- generated were calculated using an extinction coefficient of 21.1 /mM/cm at 550 nm (10) and were expressed as nmol of reduced ferricytochrome C per 10^6 cells.

MODULATION OF EXTRACELLULAR CALCIUM

Extracellular Ca^{2+} depletion was performed as previously described, with minor modifications (11). Neutrophils were suspended for 15 min at room temperature in HBSS containing 160 mM ferricytochrome C and 10 mM ethylene glycol bis (β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA; pH 7.4) (Sigma). This concentration of EGTA was shown to reduce $[Ca^{2+}]_o$ to less than 5 nM when the cells were incubated for only 2 min (11). Following a 15-minute incubation, cells were stimulated with 0.25 μM PMA and incubated at 37°C for 30 min before measuring O_2^- production. The preliminary study had shown that PMA at the concentration as low as 0.05 μM led to a respiratory burst and PMA at the concentration of 0.25μM induced the maximal response in our assay system (data not shown).

MODULATION OF INTRACELLULAR CALCIUM

Intracellular Ca^{2+} depletion was performed using a modification of a previously described procedure (12). Briefly, cells (2.5 × 10^6 cells/mL) were suspended in HBSS containing 160 mM ferricytochrome C and 1,2-bis (*o*-aminophenoxy) ethane-N,N,N′,N′-tetraacetic acid acetoxymethyl ester (BAPTA/AM; 50 or 165 μM) (Molecular Probes Inc., Eugene, OR, USA). All tubes contained same amounts of dimethyl sulfoxide (DMSO) (Sigma). Ten millimolar EGTA was also present for some samples, depending on the experiment. After a 30 min incubation at 37°C, allowing complete $[Ca^{2+}]_i$ chelation, cells were stimulated with 0.25 μM PMA for 30 min at 37°C prior to O_2^- measurement.

MEASUREMENT OF PROTEIN KINASE C ACTIVITY

PKC activity was assayed in neutrophil cytosol and membrane fractions using a modification of previously described techniques (13,14). Briefly, neutrophils were isolated and diluted to 2.5 × 10^7 cells/mL, followed by PMA-stimulation for 15 min at 37°C. Cells were then collected by centrifugation (1000 × *g*, 8 min, 4°C), snap frozen in liquid nitrogen, and stored at −70°C until the PKC assay. Cells were homogenized in 5 mL of ice cold buffer A (pH 7.4), consisting of 20 mM HEPES (Sigma), 20 mM magnesium chloride ($MgCl_2$) (Aldrich Chemical Company Inc., Milwaukee, WI, USA), 10 mM EGTA, 2 mM ethylene diamine tetraacetic acid (EDTA) (J.T. Baker Canada, Toronto, Ontario), 2 mM dithiothreitol (DTT) (Boeringer Mannheim Canada, Laval, Québec), 2 μg/mL pepstatin (Boeringer), 2 μg/mL leupeptin (Boeringer), 1 μg/mL aprotinin (Sigma) and 400 μg/mL benzamedine (Sigma). High speed centrifugation (100 000 × *g*, 60 min, 4°C) was performed to separate cytosol fraction (supernatant) from the membrane fraction (pellet). The supernatants were applied to DE52 columns (Fisher Scientific, Montréal, Québec) pre-equilibrated with buffer B (pH 7.4) consisting of 20 mM HEPES, 2 mM EGTA, 2 mM EDTA and 2 mM DTT. The membrane pellets were solubilized with ice cold buffer A with 1% (w/v) Nonidet-P40, kept on ice for 30 min, centrifuged for 30 min at 4°C (15 000 × *g*) and the supernatants were added to DE52 columns. The unbound proteins were then removed by washing the columns with buffer B and the fractions containing PKC were eluted with the same buffer containing 0.1 M sodium chloride (NaCl) (Sigma). The PKC activity in the eluates was measured by the mixed micelle assay according to Hannun et al (13). The mixed micelle reaction determined the incorporation of ^{32}P-gamma ATP (Amersham, Oakville, Ontario) into [Ser^{25}PKC] (Gibco) substrate peptide. Briefly, samples of cytosol and membrane fractions were added with reaction mixtures (1 mg/mL DTT, 34 mM NaCl, 11 mM $MgCl_2$, 30 mM HEPES, 700 μM EGTA, 700 μM EDTA, 3 mM calcium chloride ($CaCl_2$) (Sigma) and 320 μM [Ser^{25}PKC]) with or without 60 μg/mL phosphatidylserine (Avanti Polar-Lipids Inc, Alabaster, AL, USA) and 500 μg/mL diolein (Sigma) in 0.3% Triton-X 100 (BDH Inc.,Toronto, Ontario). The samples were then incubated for 10 min at 30°C and spotted onto P81 Whatmann paper (Fisher Scientific). The paper was washed using 1% phosphoric acid (J.T. Baker) followed by determination of radioactivity through scintillation counting. The protein concentrations of both cytosolic and membrane fractions were quantified by the Bradford protein assay (15).

PROTEIN KINASE C INHIBITION

Inhibition of PKC activity was performed according to the technique described by Kobayashi et al (16) and Bruns et al (17). Briefly, 1 μM calphostin C (Calbiochem, La Jolla, CA, USA) was added to the cell suspension containing HBSS and 160 mM ferricytochrome C and incubated at room temperature for 60 min under ordinary fluorescent light, in order to activate calphostin C. Neutrophils were then stimulated with 0.25 μM PMA for another 30 min at 37°C, before measurements of O_2^- generation from these cells were performed.

STATISTICAL ANALYSIS

Each individual experiment has been repeated at least 3 times. Results are presented as the mean value ± standard error of the mean (SEM) and comparisons between groups were made using the multiple comparison test (SAS).

RESULTS

EFFECT OF EXTRACELLULAR CALCIUM ON SUPEROXIDE ANION RESPONSES

The importance of $[Ca^{2+}]_o$ in O_2^- production was assessed by using the Ca^{2+} chelator, EGTA. As shown in Table I, stimulation of neutrophils with PMA resulted in a higher production of O_2^-. Furthermore, EGTA-treated cells generated O_2^- in quantities that were not statistically different from the non-EGTA-treated cells. This demonstrates that chelation

TABLE I. PMA (0.25 μM)-induced superoxide anion production in EGTA (10 mM)-treated neutrophils

Treatment	nmoles O_2^-/10^6 cells
Control	0.46 ± 0.40[a]
PMA	26.98 ± 0.86[b]
EGTA + PMA	27.30 ± 0.33[b]

[a,b] Data with different superscript letters differ at $P < 0.001$

Results are expressed as the mean ± SEM of 3 repeated experiments, each with duplicate samples

of the $[Ca^{2+}]_o$ did not have any effect on O_2^- production by PMA-activated cells. No differences could be observed between control cells and non-activated EGTA-treated cells (data not shown).

EFFECT OF INTRACELLULAR CALCIUM ON SUPEROXIDE ANION RESPONSES

The requirement for intracellular Ca^{2+} ($[Ca^{2+}]_i$) in PMA-activated cells was assessed by pre-loading the cells with different BAPTA/AM concentrations along with 10 mM EGTA. Extracellular Ca^{2+} was chelated to avoid any possible influx due to the low $[Ca^{2+}]_i$, thereby increasing free cytosolic Ca^{2+}. The comparison tests performed for O_2^- generation between BAPTA/AM (165 μM)-treated cells plus 10 mM EGTA and BAPTA/AM (165 μM)-treated cells without EGTA showed no significant differences, thus allowing the use of EGTA without influencing the effect of BAPTA/AM (Fig. 1).

A lower $[Ca^{2+}]_i$, caused by a higher BAPTA/AM concentration, led to a decreased respiratory burst induced by PMA. In fact, 50 and 165 μM BAPTA/AM resulted in a reduction of O_2^- generation by 23.0% and 88.4%, respectively (Fig. 2).

IMPORTANCE OF PKC ACTIVATION ON SUPEROXIDE ANION RESPONSES

Cytosol fractions as well as membrane fractions were isolated following PMA stimulation in order to measure PKC activity. PMA significantly increased PKC activity ($P < 0.01$) at the membrane level, not at the cytosol fraction (Fig. 3).

To further investigate the possible involvement of PKC in the response of neutrophils to PMA, the influence of the PKC inhibitor, calphostin C, on the respiratory burst was determined. As shown in Table II, a 77.26% decrease in O_2^- production occurred when the cells were pre-treated with 1 μM of the inhibitor before PMA stimulation.

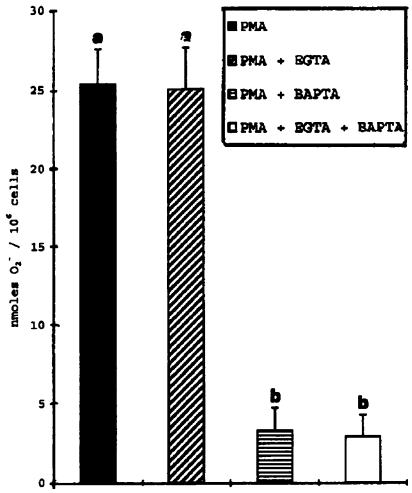

Figure 1. Effect of extracellular calcium chelation by EGTA (10 mM) on PMA (0.25 μM)-induced response of neutrophils treated with or without BAPTA/AM (165 μM). Results shown are means ± SEM of 3 independent experiments. Means with different letters are significantly different at $P < 0.001$.

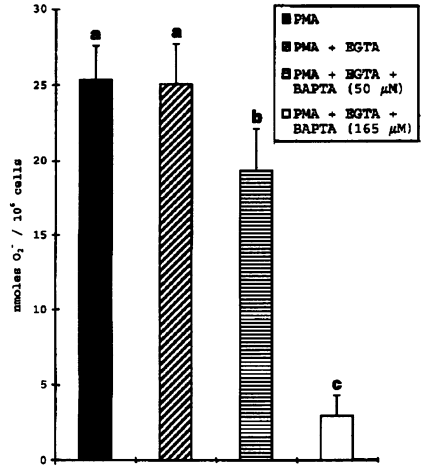

Figure 2. Superoxide anion responses of BAPTA/AM (50 or 165 μM)-loaded neutrophils after PMA (0.25 μM) stimulation. Results shown are means ± SEM of 3independent experiments. Means with different letters are significantly different at $P < 0.001$.

DISCUSSION

It has been shown that some species differences exist in terms of neutrophil biology. For example, bovine neutrophils are incapable of surmounting chemotactic responses to formyl peptides (18), although these are potent chemoattractants for neutrophils of other species (19). In addition, bovine neutrophils may lack a membrane glycoprotein analogous to the C3bi receptor of the human neutrophil (20). Bovine neutrophils have an apparent absence of lysozyme in their lysosomal granules (21). In the present study, the pathways for superoxide production after PMA stimulation were studied in bovine neutrophils.

The major finding from this study is that $[Ca^{2+}]_i$ is required for a proper O_2^- generation induced by PMA in bovine neutrophils. This differs from most results reported for human and rabbit neutrophils to some extent. PMA induced an increase in oxygen consumption without a subsequent rise in the level of $[Ca^{2+}]_i$ in both rabbit and human neutrophils (22,23).

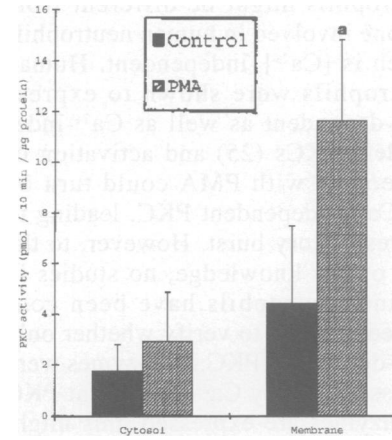

Figure 3. PKC activity of PMA (0.25 μM)-stimulated neutrophils. Results are means ± SEM of 3 independent experiments. a = $P < 0.01$ for comparison to membrane fraction of unstimulated cells (control)

TABLE II. PMA (0.25 μM)-induced superoxide anion production in calphostin C (1 μM)-treated neutrophils

Treatment	nmoles O_2^-/10^6 viable cell
Control	0.22 ± 0.17[a]
PMA	24.23 ± 2.39[b]
Calphostin C + PMA	5.51 ± 3.09[c]

[a,b,c] Data with different superscript letters differ at $P < 0.001$

Results are expressed as the mean ± SEM of 3 repeated experiments, each with duplicate samples

The rate of respiration induced by PMA at subthreshold concentrations (1 nM) could be modulated by $[Ca^{2+}]_i$ in human neutrophils, yet higher concentrations of PMA (20 nM) were

sufficient to trigger NADPH-oxidase activity independently of $[Ca^{2+}]_i$ (24). Considering the fact that the PMA concentration used in this study was sufficient to induce the maximal O_2^- response (data not shown), our results indicate that $[Ca^{2+}]_i$ in bovine neutrophils is an essential factor for O_2^- production upon PMA stimulation.

The exact reasons why bovine neutrophils are different from human and rabbit neutrophils in terms of $[Ca^{2+}]_i$ dependency for PMA stimulation are still unknown. One possibility for this difference is the requirement of $[Ca^{2+}]_i$ for the interaction between PMA and the receptor in bovine neutrophils as opposed to no Ca^{2+}-dependence for the same interaction in human neutrophils. Alternatively, the pathway implicated in the signal transduction of the respiratory burst following PMA stimulation in bovine neutrophils might be different from the one involved in human neutrophils which is $[Ca^{2+}]_i$-independent. Human neutrophils were shown to express Ca^{2+}-dependent as well as Ca^{2+}-independent PKCs (25) and activation of these cells with PMA could turn on the Ca^{2+}-independent PKC, leading to the respiratory burst. However, to the best of our knowledge, no studies in bovine neutrophils have been conducted in order to verify whether only Ca^{2+}-dependent PKC isoenzymes were xpressed. If only Ca^{2+}-dependent PKC isoenzymes are expressed, this might explain the absolute requirement for $[Ca^{2+}]_i$. Finally, variation in components of the NADPH-oxidase might exist between human and bovine neutrophils. These speculations attempting to explain the difference in $[Ca^{2+}]_i$ requirements between bovine and human neutrophils warrant further investigation.

Chelating $[Ca^{2+}]_o$ of bovine neutrophils prior to PMA stimulation did not affect their oxidative burst. These results are in accordance with those reported for human (23,26) and rabbit (22) neutrophils.

This study also demonstrated that PKC activity at the membrane level was significantly higher in PMA-stimulated cells than in the control cells. These results are in agreement with many reports on human neutrophils activated with either PMA or other phorbol esters (27–29). It is of interest to note that Wolf et al (28) mentioned that PKC activators, like PMA, regulate membrane binding of PKC as well as the enzyme activity. In fact, it was proven that the rate of membrane association of PKC in the presence of PMA and low Ca^{2+} levels is much slower than the rate obtained at much higher Ca^{2+} concentrations (29–31). Therefore, when no sufficient amounts of Ca^{2+} are found in the cells, PKC binding to the membrane and its eventual activation can be inhibited. However, these reports can not explain why PMA itself could activate PKC and induce NADPH-oxidase activation in human neutrophils even when $[Ca^{2+}]_i$ was lowered 10 to 20 times below the normal resting level (24).

To further verify whether PKC activity is essential in PMA-induced O_2^- generation by bovine neutrophils, the PKC inhibitor, calphostin C, was used. This inhibitor at 1 μM was toxic to a small percentage of neutrophils from some cows. The data has been adjusted on the viable cell basis. A significant decrease in the respiratory burst was observed following stimulation with PMA in the calphostin C-treated cells, when compared with non-stimulated cells. This confirms that activation of the PKC enzyme is essential for PMA-induced O_2^- production in bovine neutrophils. Phosphorylation of some NADPH-oxidase proteins is extremely important for the enzyme to be active and PKC appears to be highly responsible for this reaction in both human (4,32) and bovine neutrophils (33). In fact, Kramer et al (3) and Okamura et al (34) have observed a rapid phosphorylation of $p47^{phox}$, one of the components of the NADPH-oxidase, upon incubation of human neutrophils with PMA. Moreover, phosphorylation of $p47^{phox}$ was proven to be necessary for the interaction with membrane components of the oxidase (35,36), and the inactivation of the cells was accompanied by a return to the dephosphorylated state (37).

According to the similarities in the respiratory burst of our results and the numerous publications cited above, it is reasonable to speculate that bovine neutrophils require PKC phosphorylation of some of the NADPH-oxidase proteins to produce O_2^-, when stimulated with PMA. Furthermore, the fact that PKC requires Ca^{2+} for its activation could explain, to some extent, the absence of a respiratory burst obtained in the $[Ca^{2+}]_i$-depleted bovine cells.

In summary, this study showed that PMA-stimulated respiratory burst in bovine neutrophils was independent of $[Ca^{2+}]_o$, but dependent on $[Ca^{2+}]_i$. PMA was also shown to induce the respiratory burst via a PKC-dependent pathway, which is probably related to the phosphorylation and activation of some NADPH-oxidase proteins.

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